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The Research of Neutrophil Extracellular Traps in the Blood of Women with Malignization of Cervical Epithelium

Kayukova E.
Oncology Department
Chita State Medical Academy
Chita, Russia
elena_pochta22@mail.ru

Sholokhov L.
Laboratory of Physiology and Pathology o
f the endocrine system
Scientific Centre for Family Health and Human Reproduction Problems
Irkutsk, Russia
lfshol@mail.ru

Belokrinitskaya T.
Obstetrics and Gynecology Department
Chita State Medical Academy
Chita, Russia
tanbell24@mail.ru

***Abstract* — Neutrophil extracellular traps (NETs) are destroyed neutrophils combined with DNA, proteins, and enzymes; they are the destruction mechanisms of a cell attacked by pathogens. Currently its pathophysiological contribution to the neoplastic process remains controversial. The aim is to determine the number of NETs in the blood of women with cervical cells malignization. The research covered 21 cases – 7 women with cervical premalignancies (I group) and 14 women with cervical cancer of stages 0-III (II group). The average age of patients was 38±8.26. The control group was formed of volunteers without cervical pathologies (n=10). Blood serum was used as the material for the research. Neutrophils, monocytes, and NETs were counted in Feulgen stained blood smears by the optical microscope. The count was performed per 100 cells. In addition, we performed retrospective analysis of some blood parameters of the examined individuals. NETs were not found in the blood of healthy women and women with premalignancies. NETs were observed in the blood of 70% of patients with cervical cancer. The maximum number was found in the blood of patients with stage IIb cancer. Leukocytosis was registered in the blood of the patients diagnosed with cervical cancer due to neutrophilia and lymphopenia. The discovered changes increased as the neoplastic process aggravated. The results of the study and literature data point to the existing changes in blood neutrophils of the patients with cervical cancer and NETs participation in cervical carcinogenesis which requires further research.**

Keywords — cervical cancer, neutrophil extracellular traps, cancerogenesis

I. INTRODUCTION

In modern literature a new mechanism of antimicrobial activity of neutrophils, specifically formation of neutrophil extracellular traps (NETs), is widely studied. The NETs formed during the neutrophil death (NETosis) are web-like structures composed of nucleic acids, enzymes, cytoplasmic proteins, and bound pathogens, which can be represented by bacteria, viruses, tumor cells, etc. It is known that NETosis is a controlled process, which does not occur in the absence of some pathological process or condition. The traditional understanding of the pathogenetic role of NETosis was reduced to its anti-inflammatory function, which was demonstrated through elimination of certain pathogens by blocking them in traps. This was ascertained in vitro for chronic periodontitis, appendicitis, otitis, tuberculosis, mastitis, etc. [1, 2].

Excessive formation of NETs or disruption of their elimination has an anti-inflammatory effect and serves as a provoking factor for chronization of diseases. This was ascertained for some chronic immune-mediated diseases: vasculitis, systemic lupus erythematosus, rheumatoid arthritis, diabetes mellitus, atherosclerosis, etc. [3, 4].

Moreover, recently data on the dual role of NETs in relation to tumor cells have become available. There are data proving their carcinogenic as well as anticarcinogenic properties [5, 6].

Cervical cancer is traditionally viewed as an «ideal» model of carcinogenesis which reflects a stage-by-stage nature of the neoplastic process. The laboratory line of malignant cervical cancer cells (HeLa cells) is of the highest demand in medical experiments [7].

Therefore, research of NETosis makes it possible to provide insight into previously unknown mechanisms of carcinogenesis of such severe pathological condition as cervical cancer.

II. The Aim of the Research

The aim of the research is to determine the amount of NETs in the peripheral blood of the patients with cervical cancer, and to determine their interrelation with malignization of cervical epithelium in order to develop pathogenetically substantiated methods of its prevention and treatment.

III. Material and Methods

We did not find any statistically significant differences in the studied parameters in patients with premalignancies. Blood smears of only 2 women from the I group contained 1 and 5 NETs respectively (Table 1).

From each woman 5 ml of peripheral blood was collected, settled for 30 minutes, and centrifuged at 37°C and 1500 rotations per minute for sedimentation of erythrocyte suspension. Neutrophils, monocytes, and neutrophil extracellular traps were counted in Feulgen stained blood smears by the optical microscope. The count was performed per 100 cells. In addition, we performed retrospective analysis of some blood parameters of the examined individuals (white blood cell count, neutrophils, lymphocytes, and monocytes). The results were analyzed by nonparametric statistical analysis with application of Mann–Whitney U test. The differences were considered statistically significant at $p<0.05$.

IV. Results

We did not find any statistically significant differences in the studied parameters in patients with premalignancies. Blood smears of only 2 women from the I group contained 1 and 5 NETs respectively (Table 1). In the blood of patients with cervical cancer (II group) leukocytosis was registered due to increase in the share of neutrophil granulocytes by 35 % in comparison with the control group ($p\leq0.05$), and decrease in the number of lymphocytes by 29.2 % ($p\leq0.05$). Against this background neutrophil to lymphocyte ratio in the II group doubled ($p\leq0.05$).

The blood of 70% of the patients diagnosed with cervical cancer contained NETs microscopically determined as cloud-like structures composed of chromatinic filaments wrapped around tumor cells (Figure 1).

Fig. 1. Neutrophil Extracellular Trap in the Blood of the Patient with Cervical Cancer (Feulgen-Stained Blood Smear)

As the neoplastic process aggravates, the total count of leukocytes in the blood of the patients increases mainly because of increased share of neutrophils ($p<0.05$). The maximum count of granulocytic neutrophils was registered in the patients diagnosed with stage III cervical cancer – 71.5 [66.3; 67.5], which is 20.8% more than in case of stage IIb cervical cancer ($p\leq0.05$) (Table 2).

It is interesting to note that the changes observed in the leukograms of the patients diagnosed with cervical cancer occur against the background of lymphopenia ($p<0.05$). Thus, the count of lymphocytes in the blood of the patients diagnosed with stage III cervical cancer is by 45% less in comparison with the same at stage 0 of cervical cancer ($p<0.05$). This fact is also confirmed by a threefold increase of neutrophil to lymphocyte ratio in this category of patients ($p<0.05$).

The NETs were visualized in the blood smears of all cervical cancer subgroups irrespective of the disease stage. Their maximum quantity was found in the blood of the patients with stage IIb– from 4 to 13 in the field of vision. Particularly in this subgroup NETs were detected in virtually all cases (90 %).

TABLE I. Indicators of Leukogram and NETosis in the Peripheral Blood of the Patients with Premalignancies and Cervical Cancer

group / index	control (n=10)	I group (n=10)	II group (n=34)	p
WBC, 10^9/l	6.06 [6.06; 7.22]	5.81* [5.67; 5.94]	9.48* [8.03; 9.86]	**p1≤0.01**
NEU, %	45.8 [42.1; 55.78]	52.0 [51.53; 52.35]	61.75* [59.1; 71.5]	p1>0.05
LYM, %	42.1 [1.04; 43.45]	33.3 [33.3; 34.7]	29.8* [18.1; 29.8]	**p1≤0.05**
NLR	1.04 [1.04; 1.65]	1.57 [1.48; 1.57]	2.08* [2.08; 9.19]	p1>0.05
MON, %	7.5 [6.98; 8.1]	9.3 [9.0; 10.1]	7.8* [6.33; 9.08]	p1>0.05
NET, abs.	-	0	3* [0.25; 4]	p1>0.05
The number of women in whose blood NETs are detected, %	-	20%	70%*	**p1≤0.05**

[a.] Remarks

* significant differences between the corresponding group and the control group

p1 - significant differences between I and II groups

Abbreviations

WBC - white blood cell count, NEU - total neutrophil count, LYM - total lymphocytecount, MON - total monocyte count

TABLE II. INDICATORS OF LEUKOGRAM AND NETOSIS IN THE PERIPHERAL BLOOD OF PATIENTS WITH CERVICAL CANCER

group / index	in situ (n=7)	stage Ia1 (n=8)	stage IIb (n=9)	stage IIIb (n=10)	p
WBC, 10^9/l	5.9 [5.65; 6.49]	6.11 [5.8; 6.8]	8.03 [8.03; 18.9]	9.48 [6.97; 9.48]	**p1 ≤ 0.01** p2 ≥ 0.05 **p3 ≥ 0.01** **p4 ≤0.05**
NEU, %	54.6 [53.4; 56.8]	61.0 [58.0; 62.7]	56.6 [56.6; 80.5]	71.5 [66.3; 67.5]	**p1 ≤ 0.01** p2 ≥ 0.05 p3 ≤0.05 p4 >0.05
LYM, %	33.0 [31.4; 34.6]	29.8 [27.2; 30.8]	30.3 [5.9; 30.3]	18.1 [18.1% 18.1]	**p1 ≤ 0.01** p2 ≥ 0.05 p3 ≤0.05 p4 >0.05
NLR	1.67 [1.59; 1.72]	0.09 [0.08; 0.1]	1.87 [1.87; 13.6]	3.66 [3.66; 3.73]	p1 ≥ 0.05 **p2 ≤ 0.01** **p3 ≤0.05** p4 >0.05
MON, %	8.7 [7.75; 10.3]	6.3 [5.2; 6.33]	7.9 [2.3; 8.6]	8.6 [2.3; 7.9]	**p1 ≤ 0.01** p2 ≥ 0.05 **p3 ≤0.05** p4 >0.05
NET, abs.	3.0 [1.5; 5.5]	1.5 [0; 3]	4.0 [4; 13]	1.0 [1.0; 1.0]	p1 ≥ 0.05 **p2 ≤ 0.01** **p3 ≥ 0.01** p4 >0.05
The number of women in whose blood NETs are detected, %	70%	75%	88.9%	80%	p1 ≥ 0.05 **p2 ≤ 0.01** **p3 ≥0.05** p4 >0.05

Remarks

* significant differences between the corresponding group and the control group

p1 - significant differences between stage 0 and stage Ia1 cervical cancer

p2 - significant differences between stage Ia1 and stage IIb cervical cancer

p3 - significant differences between stage IIb and stage IIIb cervical cancer

p4 - significant differences between stage IIIb and stage 0 cervical cancer

Abbreviations

WBC - white blood cell count

NEU - total neutrophil count

LYM - total lymphocyte count

MON - total monocyte count

V. DISCUSSION

Currently new data on the pathophysiological role and types of NETosis in the pathogenesis of many diseases have become available.

Previously only data on suicidal NETosis were available, which involved formation of NETs as a final stage of neutrophil death; the pathophysiological function of NETosis was to block and remove enthetic agents.

Nowadays another type of NETosis - vital NETosis - is distinguished, where NETs are formed through vesicular exocytosis, the membrane remains intact and the cell viable. Moreover, the information on another type of NETosis became available, whereby NETs are formed through the release of mitochondrial DNA (instead of nuclear DNA and neutrophil granules), while their structure remains unchanged as well.

The expansion of information on the pathogenetic role of NETs indicate their multimodal significance in the development of many diseases, including oncological ones.

The cancerogenic action of NETs can be conventionally combined in the following groups of mechanisms.

First - they activate proliferation of tumor cells. McGarry Houghton et al. (2013) established that proliferative signal pathway PI3K/AKT/mTOR is activated in lung adenocarcinoma of mice under the influence of elastase, one of the enzymes contained in neutrophil granules [8].

It was determined in vitro that NETs cause activation of TOLL-dependent signal pathways, which stimulate proliferation and metastatic spreading of cancer cells [9].

Secondly, they induce metastatic spreading. Enzymes released into intercellular space during formation of NETs, such as elastase and proteinase potentiate epithelial–mesenchymal transition of NET-tumor cells complexes, thus,

initiating their dissemination [10, 11]. When they enter the blood flow, circulating tumor complexes against the pro-flammatory cytokine background associated inter alia with NETosis, form complexes with thrombocytes contributing to the survival of the latter, extravasation through mesenchymal–epithelial transition, and formation of metastatic niches [12, 13].

Thirdly, they cause dysfunction of organs. NETosis contributes to vascular dysfunction and systemic inflammation in organs which are not tumor growth areas, such as heart and kidneys, provoking the development of dysfunction of organs, which was confirmed in vitro. NETosis provoked development of cardiovascular insufficiency and kidney failure in mice with breast carcinoma and insulinoma [12].

Fourthly, they induce thrombosis. NETs are procoagulants and can stimulate tumor growth through the local increase of thrombin concentration, and activation of intrinsic pathway of coagulation. Besides, NETs activate thrombocytes. Currently NETs are one of the reasons of thrombosis in oncology patients and are viewed as a potential target for its prevention [8, 12].

Fifthly, they stimulate angiogenesis. NETs contain and activate matrix metalloproteinase (MMP-9), collagenase, elastase, and angiogenic factor VEGF that stimulate angiogenesis [8, 14].

And finally, they cause immunodeficiency. NETs suppress cytotoxic antitumor response CD8+. NETs modify immunosuppressive phenotype of T-lymphocytes, characterized by hyperexpression of PD-L1 and IL-10 [8, 15].

Currently attempts to affect NETs have been made in vitro as a method to block tumor dissemination. The treatment of NETs by PAD-4 inhibitor (peptidyl arginine deiminase type IV causes decondensation of chromatin and its release out of nucleus) resulted in decreased NET formation in experimental animals [8]. The treatment of NETs by deoxyribonuclease I (DNAse I) in mice decreased intensity of breast cancer cell migration and prevented lung metastasis [16].

M.C. Hawes et al. (2015) in literature review refer to the results of experimental research, which proved clinical efficiency of DNAse 1 for many cancer cell lines: overall survival growth due to inhibition of metastatic spreading [17].

The factors, which initiate NETosis in oncology patients are still being studied. It is known that proinflammatory cytokines, for example granulocyte colony-stimulating factor (G-CSF) serve as its inductor, which promotes attraction of neutrophils and causes their activation. It was found that tumors with high systemic level of G-CSF show higher intensity of NETosis, and less favorable prognosis. In addition, interleukine-8 is now also viewed as a NETosis-inducing agen [18]. This fact is consistent with the previously obtained data on high level of proinflammatory cytokines in systemic circulation of patients diagnosed with cervical cancer [19].

Above that, tumoral environment can secrete other inductors of NETosis, such as TNF-α, interleukine-1, and CXCL2 [8].

According to some data, cancer cells can regulate intensity of NETosis. Tumor cells inhibit apoptosis of neutrophils, thus causing prolonged duration of their life as shown in vitro for gastric cancer, cervical cancer, head and neck cancer, and colorectal cancer. Above that, it was found that neutrophils in the peripheral blood of oncology patients can be less mature and have a longer life [14].

Available data indicate that NETs formation is a controlled process. We are beginning to realize, that tumor itself and its microenvironment regulate NETosis.

Our data on pro-cancerogenic role of NETs in cervical oncogenesis are consistent with the results of other studies. In particular, Fomenko Yu. et al. (2018) detected NETs formed via suicidal NETosis in the blood of patients with cervical cancer and determined, that their quantity varies after radiation therapy [20].

The changes discovered in the leukogram of the patients suffering from cervical cancer and leukocytosis with lymphocytopenia are consistent with some results of other experimental studies. According to literature data, neutrophil leukocytosis and increased neutrophil to lymphocyte ratio in the peripheral blood of oncology patients is an unfavorable predictive factor, which reflects aggressive growth of tumor and unfavorable disease prognosis. This fact was determined for kidney cancer, melanoma, colon cancer and rectal cancer, hepatocellular carcinoma, etc. According to some data, the value of this parameter can be taken into consideration as a predictor of tumor response to neoadjuvant chemotherapy, in particular, for esophageal cancer the frequency of objective responses to neoadjuvant chemotherapy was higher in the group of patients with neutrophil to lymphocyte ratio less than 2.2. [14]

Lin Wang et al. (2017) determined that increased neutrophil to lymphocyte ratio in the peripheral blood of the patients diagnosed with cervical cancer correlates with the frequency of lymph node metastasis. However, these changes are not characteristic of early cervical cancer [21].

VI. CONCLUSIONS

1. NETs were not found in healthy people.

2. Peripheral blood of the patients with cervical premalignancies did not show any statistically significant differences by studied parameters.

3. In the blood of the patients diagnosed with cervical cancer leukocytosis was registered due to neutrophilia and lymphopenia. The discovered changes increase as the neoplastic process aggravates.

4. The neutrophil to lymphocyte ratio in the peripheral blood was higher in the patients diagnosed with cervical cancer.

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